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Systems Biology Approach Predicts Antibody Signature Associated with *Brucella melitensis* Infection in Humans

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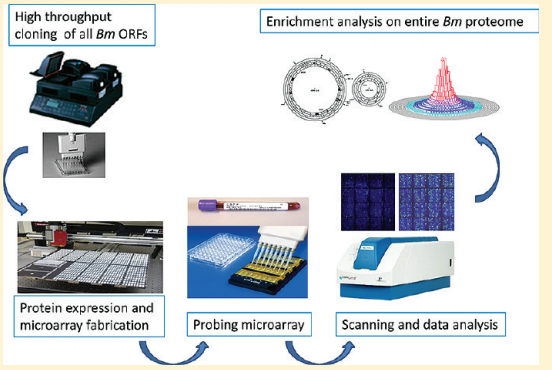
Supporting Information

ABSTRACT: A complete understanding of the factors that determine selection of antigens recognized by the humoral immune response following infectious agent challenge is lacking. Here we illustrate a systems biology approach to identify the antibody signature associated with *Brucella melitensis* (*Bm*) infection in humans and predict proteomic features of serodiagnostic antigens. By taking advantage of a full proteome microarray expressing previously cloned 1406 and newly cloned 1640 *Bm* genes, we were able to identify 122 immunodominant antigens and 33 serodiagnostic antigens. The reactive antigens were then classified according to annotated functional features (COGs), computationally predicted features (e.g., subcellular localization, physical properties), and protein expression estimated by mass spectrometry (MS). Enrichment analyses indicated that membrane association and secretion were significant enriching features of the reactive antigens, as were proteins predicted to have a signal peptide, a single transmembrane domain, and outer membrane or periplasmic location. These features accounted for 67% of the serodiagnostic antigens. An overlay of the seroreactive antigen set with proteomic data sets generated by MS identified an additional 24%, suggesting that protein expression in bacteria is an additional determinant in the induction of *Brucella*-specific antibodies. This analysis indicates that one-third of the proteome contains enriching features that account for 91% of the antigens recognized, and after *B. melitensis* infection the immune system develops significant antibody titers against 10% of the proteins with these enriching features. This systems biology approach provides an empirical basis for understanding the breadth and specificity of the immune response to *B. melitensis* and a new framework for comparing the humoral responses against other microorganisms.



KEYWORDS: *Brucella melitensis*, enriching features, immune response, protein microarray

INTRODUCTION

A major component of the adaptive immune response to infection is the generation of protective and long-lasting humoral immunity, but factors governing selection of the particular antigens recognized are unclear.[1,2] It is not uncommon for viruses encoding a small number of proteins to generate antibodies against each encoded protein. But for infectious agents containing hundreds or thousands of proteins only a subset of the proteome is recognized and little is known about the extent or the characterisitics of this subset of antigens. Methods for making a complete empirical accounting of the immunoproteome have limitations, particularly when the genome of the organism is large. Here we describe a *B. melitensis* proteome microarray that enables this problem to be directly addressed by applying an unbiased systems biology approach to identify immunodominant

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and serodiagnostic antigens and to classify the reactive antigens based on functional and physical properties.

Protein microarrays can be used with relative ease to probe the entire proteome of different infectious microorganisms including bacteria, viruses and parasites.[3−13] This approach permits assessing the repertoire of antibodies produced in response to infections or vaccinations from large collections of individual patient sera, and can be used to perform large-scale sero-epidemiological and sero-surveillance analyses not possible with other technologies, while consuming small quantities (<2 μL) of each serum sample. Probing large numbers of patient specimens empowers the statistical tests resulting in more reliable conclusions while correcting for false discovery required for multiple comparison testing inherent in microarray analysis.[3,5,7,9] Moreover, microarrays can display all proteins of an infectious agent and may allow for identification of novel antigens, otherwise undetectable by methods like 2-D gels that are highly biased by microbial protein expression patterns.

Brucellosis is a worldwide zoonosis caused by bacteria of the genus *Brucella*. *Brucella melitensis*, the most virulent species infecting humans, also infects goats, sheep and cattle,[14−16] in Central and South America, the Middle East, East Asia, and some southern European countries.[17,18] Consumption of animal products, direct animal contact and inhalation of aerosolized bacteria in the laboratory setting (indicating the potential for air-borne spread via a bioterrorism-type attack) are well-recognized routes of infection. The current knowledge of protein antigens recognized by humans and reservoir animals is based on limited numbers of studies on *Brucella melitensis* and *Brucella abortus*.[19−31]

We recently constructed a pilot proteome array consisting of 1406 *B. melitensis* proteins and observed marked differences in immune responses between humans and goats.[5] Here we have expanded our previous study to the full proteome microarray consisting of 3046 *B. melitensis* proteins, by including expression of newly cloned 1640 *Bm* genes. In addition to developing a better classifier capable of predicting disease based on serological response, more importantly, this study allows an estimate of the extent of immune reactivity against the whole proteome, and prediction of proteomic features that may dictate immune recognition for other yet uncharacterized gram-negative bacteria.

MATERIALS AND METHODS

Ethics Statement

Human sera were obtained from patients enrolled in a prospective clinical study of brucellosis in Lima, Peru. The human subjects part of the study was approved by the Humans Research Protections Committee of the University of California San Diego, the Comite de Ética of Universidad Peruana Cayetano Heredia, Lima, Peru and the Comite de Ética of Asociación Benéfica PRISMA, Lima, Peru, all of whom have maintained federal wide assurances with the United States Department of Health and Human Services. All patients provided written informed consent prior to enrollment in the study, and signed consent forms have been stored in locked files in study offices at UPCH and AB PRISMA, Lima, Peru.

Human Serum Samples

Human sera were obtained from patients in Lima, Peru. All patients in this studywere infected with *B. melitensis* biovar 1. Forty- two patients were confirmed to have acute brucellosis by positive culture, positive Rose Bengal test and by tube agglutination tests titers ≥1/160. Eighteen patients presenting with brucellosis-compatible syndromes were culture negative and Rose Bengal positive, and treated according to standard antibiotic therapy within 2 days of serum sampling. Additional control patient samples included 13 sera from Rose Bengal-negative patients, 44 samples from ambulatory healthy controls from north Lima where brucellosis occasionally affects patients, and sera from humans in the U.S. where brucellosis is not found.

Brucella melitensis Lipopolysaccharide Purification

Approximately 10 g of autoclaved pellet of *B. melitensis* 16 M were used to isolate LPS using the hot phenol-water method.[32] The purified LPS was treated with RNase, DNase and Proteinase-K, and the hot phenol-water treament was repeated. *B. melitensis* LPS obtained from both upper phenol saturated aqueous layer (aqueous phase) and lower water saturated phenol layer (phenol phase) were pooled. LPS from *E. coli* 055:B5 was purchased from Sigma Chemicals (St. Louis, MO). The LPS from *E. coli* 055:B5 and *B. melitensis* 16 M were analyzed on a gradient 4−12% Tris-Glycine sodium dodecyl sulfate (SDS) polyacrylamide gel (Invitrogen Corp., Carlsbad, CA) under reducing conditions. The presence of LPS in the gels was detected with a periodic acid silver stain[33] and protein using Coomassie blue stain. *B. melitensis* LPS was quantified using a colorimetric assay to measure 2-keto-3-deoxyoctonate (KDO) concentration.[34] *E. coli* 055: B5 LPS (Sigma, St. Louis, MO) was used as standard.

Microarray Fabrication, Probing and Immunostrips Probing

All ORFs from *Brucella melitensis* 16 M genomic DNA were identified using GenBank NC_003317 and NC_003318, and 1640 ORFs that were absent from pilot chip were amplified and cloned using a high-throughput PCR and recombination cloning method described previously.[5] Microarrays and immunostrips were fabricated and probed as described before.[5] Plasmids were expressed at 24 °C for 16 h in *in vitro* transcription/translation *E. coli* reactions (Expressway Maxi kits from Invitrogen), according to the manufacturer's instructions. For microarrays, 10 μL of reaction was mixed with 3.3 μL 0.2% Tween 20 to give a final concentration of 0.05% Tween 20, and printed onto nitrocellulose coated glass FAST slides (Whatman) using an Omni Grid 100 microarray printer (Genomic Solutions). For immunostrips, *B. melitensis* LPS was printed at 0.01 mg/mL. All antigens were printed on Optitran BA-S 85 0.45 μm Nitrocellulose membrane (Whatman) using BioJet dispenser (BioDot) at 1 μL/cm, and cut into 3 mm strips. Human sera samples were diluted to 1:200 with 10 mg/mL *E. coli* lysate (Mclab). Microarray slides were incubated in biotin-conjugated secondary antibody (Jackson ImmunoResearch) diluted 1/200 in blocking buffer, and detected by incubation with streptavidin-conjugated SureLight P-3 (Columbia Biosciences). The slides were washed and air-dried by brief centrifugation. Microarray slides were scanned and analyzed using a Perkin-Elmer ScanArray Express HT microarray scanner. Intensities were quantified using QuantArray software. All signal intensities were corrected for spot-specific background. For immunostrips probing, human sera were diluted to 1/200 in 5% BSA solution containing 20% *E. coli* lysate (McLab). Strips were then incubated in alkaline phosphatase conjugated donkey antihuman immunoglobulin (anti-IgG, Fcγ fragment-specific, Jackson ImmunoResearch) secondary antibody, diluted to 1/2000, and reactive bands were visualized by incubating with 1-step Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt (NBT/BCIP) developing buffer (Thermo Fisher Scientific). Immunostrips were scanned with Hewlett-Packard document scanner, and were quantified using Image J software.

Mass Spectrometry Data Set from Pathogen Portal Web Site

The mass spectrometry data set "characterization of host and pathogen proteins affected by *Brucella abortus* infection" was from Caprion Proteomics studies of *B abortus*, downloadable from pathogen portal web site hosted by proteomics resource center at http://www.pathogenportal.org/portal/portal/PathPort/Home. Samples were processed for "intact bacteria" or "cell envelope" preparations from exponentially growing bacteria according to the Caprion protocol 24401 and 24302, as described before.[23,35] Briefly, to process intact bacteria for MS analysis,

50 μL aliquots of 20 μg protein samples were denatured in 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO) containing 8 M urea. After sonication, samples were centrifuged at 2000× *g* for 1 min and a 950 μL cold (−20 °C) chloroform/methanol solution (2:1 v/v) were added. Samples were vortex mixed and incubated at −20 °C for 2 h. Subsequently, 100 μL cold (−20 °C) methanol was added and samples were clarified by centrifugation at 21 000× *g* for 10 min at 4 °C. The supernatants were dried under vacuum, resuspended in 100 mM ammonium bicarbonate containing 8 M urea and 1% (w/v) of acid-labile surfactant (ALS; Waters, Milford, MA) and sonicated for 10 min. After centrifugation for 20 s at 2000× *g*, samples were incubated for 1 h at ambient temperature. A volume of 450 μL of 100 mM ammonium bicarbonate containing 5% (v/v) of acetonitrile was added. Lys-C (Wako, Richmond, VA) was added to yield a 1:50 enzyme to protein ratio. The samples were incubated for 3 h at 37 °C. Trypsin (Promega, Madison, WI) was then added at a 1:50 enzyme to protein ratio and samples were incubated for an additional 16 h. Following proteolysis, TCEP (Pierce, Rockford, IL) was added to a final concentration of 10 mM and samples were incubated for 30 min at ambient temperature and then lyophilized. To cleave the acid labile surfactant, samples were incubated for 30 min at ambient temperature in 100 μL of 1 N HCl and 100 μL of water were added into each sample with subsequent incubation for another half hour at ambient temperature. To process cell envelope for MS analysis, supernatants of exponentially growing *B. abortus* strains were clarified, centrifuged at 100 000× *g* for 6 h at 4 °C, pellets resuspended in double distilled water. The sample was sonicated, and 50 mM ammonium bicarbonate containing 2% acid-labile surfactant and 8 M urea were added. The sample was vortex mixed for 1 h. A sample of 50 μL of the OM suspension was added to a final volume of 1 mL of a chloroform/methanol solution (2:1 v/v). The sample was vortex mixed and incubated at −20 °C for 2 h. Subsequently, 100 μL of cold methanol (−20 °C) was added, and the sample was cleared by centrifugation for 10 min at 21 000× *g*. The supernatant was dried under vacuum and resuspended in 4 M urea; 50 mM ammonium bicarbonate pH 8.0. Lys-C was added at a 1:50 protein ratio. The samples were then diluted 4:1 with 50 mM ammonium bicarbonate buffer pH 8.0, and trypsin was added at a 1:25 protein ratio for an additional 16 h. Following proteolysis, the samples were distributed into 96-well plates for mass spectrometry analysis.

Peptide digests were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) as described.[23,35,36] The LC−MS system consisted of a CapLC (Waters, Milford, MA) with a cooled autosampler and a QTOF Ultima (Waters, Milford, MA) controlled by MassLynx version 4.0 software. The peptide concentrations were normalized and the samples were injected into a reversed-phase column (Jupiter C18, Phenomenex, Torrance, CA) for HPLC separation. Protein identification was done by submitting LC−MS spectra to Mascot software (MatrixScience, Boston, MA) for searching against the National Center for Biotechnology Information protein database (NCBI). The parameters used for Mascot search and protein homology clustering were previously detailed.[23,35] Numbers of different peptides for each identified protein were counted and listed in Table S4 (Supporting Information).

Data Analysis

Data analysis was performed using the R (http://www.r-project.org) and SAS (http://www.sas.com/) statistical software. It has been noted in literature that data derived from microarray platforms is heteroskedatic.[37−39] To stabilize the variance,[40,41] the vsn normalization method implemented as part of the Bioconductor suite (www.bioconductor.org) was applied to the quantified array intensities. In addition to removing heteroskedacity, this procedure corrects for nonspecific noise effects by finding maximum likelihood shifting and scaling parameters for each array such that control probe variance is minimized. This calibration has been shown to be effective on a number of platforms.[42]

Differentially reactive proteins between groups were determined using a Bayes regularized *t* test adapted from Cyber-T for protein arrays,[37,43] which has been shown to be more effective than other differential expression techniques.[44] To account for multiple comparison conditions, the Benjamini and Hochberg (BH) method was used to control the false discovery rate.[45] After Benjamini and Hochberg correction, *p*-value smaller than 0.05 was considered significant, and the corresponding protein was considered differentially reactive and serodiagnostic. Multiplex classifiers were constructed using linear and nonlinear Support Vector Machines (SVMs) using the "e1071" R package. Plots of receiver operating characteristic (ROC) curves were made with the "ROCR" R package. Sensitivity specificity and Area Under the Curve (AUC) were determined from the resulting ROC curves. Principle Component Analysis (PCA) to visually reduce and summarize signal intensity variance among the subjects was conducted in JMP software (www.JMP.com).

Hierarchical clustering was used to group sera samples into subsets, such that those within each cluster (subset) are more closely related to one another than samples assigned to other clusters. Clustering is based on the degree of similarity between the protein intensity for each individual. MeV v4.6 (TM4Microarray Software Suite, www.tm4.org) was used to perform the clustering analysis.

The following programs were utilized for computational prediction. TMHMM v2.0 was utilized for transmembrane domains prediction[46] (http://www.cbs.dtu.dk/services/TMHMM/), SignalP v3.0 for signal peptide prediction[47] (http://www.cbs.dtu.dk/services/SignalP/), PSORTb v3.0. software for cellular location prediction[48] (http://www.psort.org/psortb/). PI/MW tool from Swiss Institute of Bioinformatics was used to determine isolectric point (http://ca.expasy.org/tools/pi_tool.html). P value for enrichment statistical analysis was calculated using Fisher's exact test in the R environment.

RESULTS

Gene Amplification, Cloning and Protein Expression

The 3198 predicted ORFs of *B. melitensis* (*Bm*) 16 M were subjected to amplification from genomic DNA, and 1406 ORFs were cloned and printed on pilot chip.[5] In this study, another 1640 ORFs were successfully cloned using our high throughput recombination method. About one-fourth of the cloned genes

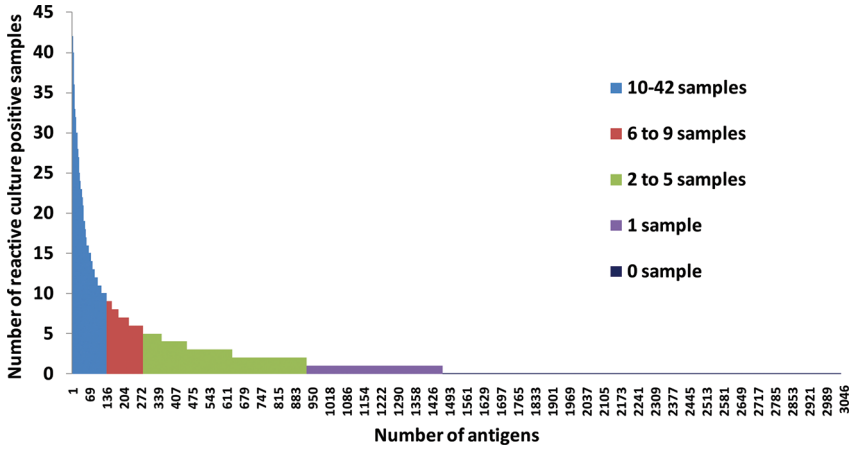

Figure 1. Reactivity of the Immunoproteome. Each bar represents one of the 3046 proteins of *B.melitensis.* The bar height reflect the number of sera reactive to each protein. Nonreactive proteins account for approximately 52% of the proteome. The raised bars of any color represent 1464 reactive proteins, which define the immunoproteome (approximately 48% of the proteome). There are 538 antigens reactive in 1 sample, 648 antigens reactive in 2−5 samples, 143 antigens reactive in 6−9 samples and 135 antigens reactive in 10−42 samples. BMEI0536 and BMEI1079 reacted in all 42 *Bm* culture positive samples. All serodiagnostic antigens except BMEI0503 reacted in 10−42 samples; BMEI0503 reacted in 9 samples.

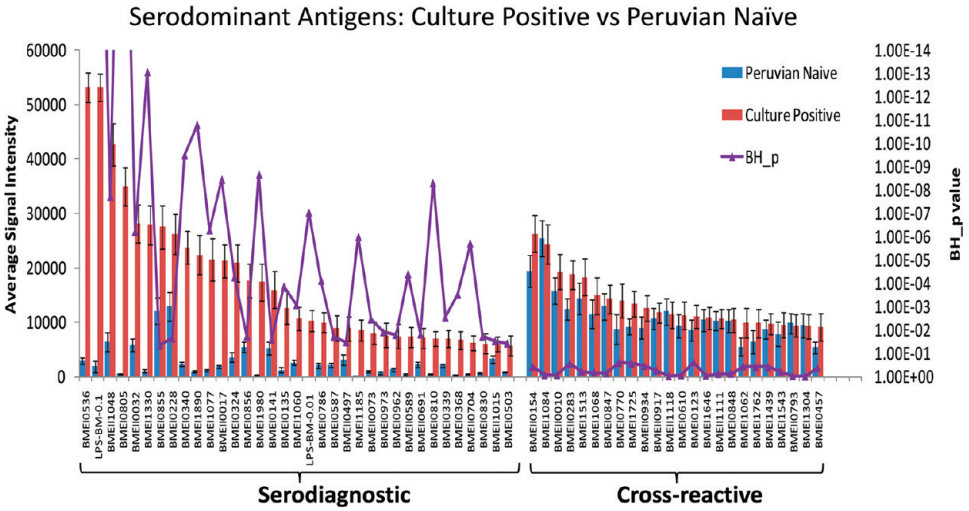

Figure 2. Discovery of new human serodiagnostic antigens by probing the full *B. melitensis* proteome arrays with a collection of *B. melitensis* Peruvian naive and culture positive human sera. The mean sera reactivity of the 3046 antigens was compared between the culture positive and Peruvian naive groups. Antigens with Benjamini Hochberg corrected *p*-value less than 0.05 (serodiagnostic) are organized to the left and cross-reactive (BH_*p* > 0.05) antigens to the right. Shown are the 33 serodiagnostic protein antigens, *Bm* LPS, and a subset of cross-reactive antigens.

were sequenced and >99% of sequenced clones had the correct sequence. All 3046 *Bm* ORFs cloned in pXT7 vector (>95% of proteome) were expressed under T7 promoter in the *E. coli in vitro* transcription/translation system, and printed on microarrays. Over 98% of protein spots were confirmed positive for expression (Figure S1A, Supporting Information).

Human Antibody Profile

Bm protein arrays were probed with sera from acute brucellosis patients in Lima, Peru obtained within 1−3 weeks of the onset of symptoms, and sera from *Bm* culture-positive humans (Figure S1B, Supporting Information) showed robust reactivity against a collection of antigens compared to unexposed individuals. Among the 3046 antigens tested, 1464 antigens reacted with at least one culture positive individual, accounting for 48% of the proteome (Figure 1). Within this immunoproteome, 122 protein antigens were defined "serodominant", with mean reactivity greater than the mean of the no DNA controls plus 2.5 standard deviations among culture positive individuals (Figure 2, Figure S2 and Table S1, Supporting Information). Of these, 33 protein antigens were serodiagnostic, and were significantly differentially reactive between naïve and culture positive patients from Peru (Benjamini and Hochberg adjusted Cyber-T *p*-value <0.05). Most of these antigens also reacted strongly with sera from brucellosis cases that were culture negative but positive for the Rose Bengal diagnostic test. An unbiased hierarchical clustering analysis segregated most of the individual specimens into two groups, with a few infected cases with weak reactivity sporadically clustered among samples diagnosed as uninfected (Figure S2, Supporting Information). Peruvian naïve samples exhibited very low responses to this collection of antigens. We also identified 90 cross-reactive antigens that reacted similarly among all human

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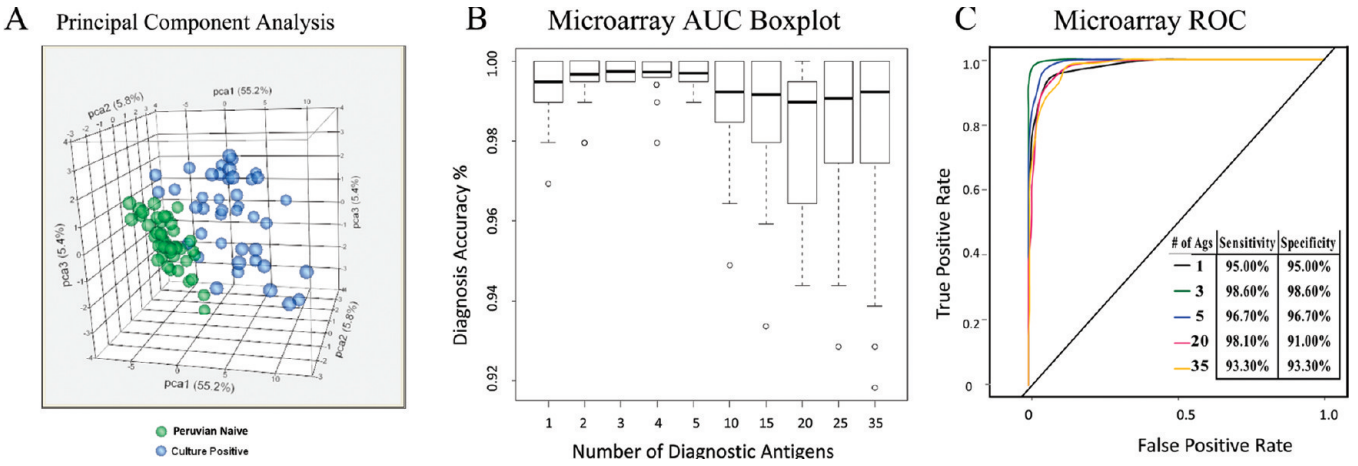

Figure 3. Statistical presentation of principal component analysis and multiple antigens AUC. (A) Unsupervised principal component analysis of the signal intensity for samples from Peruvian naives and culture positive groups revealed that these two groups could be segregated on the basis of 33 selected serodianosis protein antigens and 2 *Bm* LPS (at 0.1 mg/mL or 0.01 mg/mL). The serodiagnostic antigens were ranked by single antigen AUC. (B) Cross-validation AUC boxplot with variance and mean value and (C) cross-validation ROC graphs show classifiers with increasing number of human serodiagnostic antigens. With the top 3 antigens, this classifier yielded a highest sensitivity and specificity rate of 98.6% and 98.6%, respectively. All 33 antigens plus *Bm* LPS also produced sensitivity and specificity over 93%.

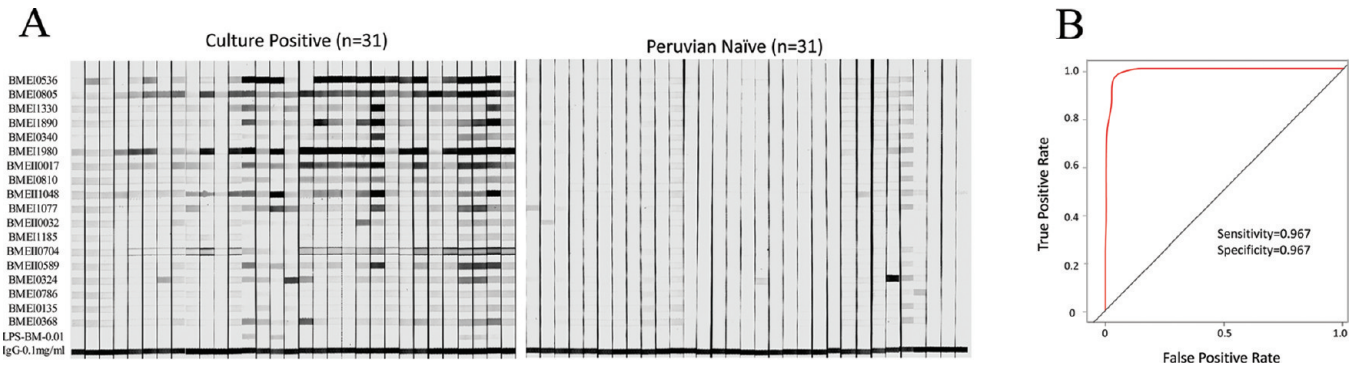

Figure 4. Immunostrips probing. (A) Eineteen serodiagnostic antigens and *Bm* LPS at 0.01 mg/mL were printed onto nitrocellulose membrane in adjacent stripes using a BioDot jet dispenser as described in Materials and Methods. Strips were probed with culture positive or Peruvian naive sera diluted 1/200 followed by alkaline phosphatase conjugated secondary antibody and enzyme substrate. Weak reactivity in the naïve healthy controls can be distinguished from the strong reactivity in the infected group. (B) Cross-validation ROC curve was generated and sensitivity and specificity of immunostrips test is 96% and 96%, respectively.

samples, whether from naïve individuals or individuals diagnosed to be infected.

Classifier of Serodiagnostic Antigens

We performed unsupervised principal component analysis and showed that the 33 serodiagnostic antigens clearly separated the Peruvian naive from culture positive groups (Figure 3A). To further determine the accuracy of distinguishing brucellosis cases from controls, cross-validation receiver operating characteristic (ROC) curves and area under the curve (AUC) boxplots were generated (Figure 3). The serodiagnostic antigens were ranked by decreasing single antigen AUC. The top four ORFs, BMEI0536 (bp26), BMEI0805 (hypothetical protein), BMEI1330 (protease Do), and BMEI 1890 (transporter), as wells as *Bm* LPS, all have an individual antigen AUC greater than 0.90 (Table S1, Supporting Information). Three of the antigens were identified previously on microarrays.[5] Antigen bp26 (BMEI0536; AUC 0.991; BH p-value $< 1 \times 10^{-16}$) gave the best single antigen discrimination with 95% sensitivity and specificity rate. We used kernel methods and support vector machines[43,49] to build linear and nonlinear classifiers. Variability and mean value of accuracy from this model has been shown in the AUC boxplot. The top 5 antigens all produced sensitivity and specificity over 96% (Figure 3C). With the top 3 antigens, this classifier yielded a highest sensitivity and specificity rate of 98.6 and 98.6%, respectively. Altogether 33 protein antigens plus LPS produced sensitivity and specificity over 93%.

Validation of Serodiagnostic Accuracy with Immunostrips

Eighteen serodiagnostic proteins and *Bm* LPS were printed onto nitrocellulose membranes using a BioDot jet dispenser. The membrane was then cut into 3 mm strips. The individual strips were probed with 31 different randomly selected culture positive sera and 31 Peruvian naive sera. Brucellosis patients reacted strongly against the serodiagnostic antigens with variable signal intensities among the patients. Naïve samples showed much lower reactivity against these serodiagnostic antigens (Figure 4A). The ROC curve (Figure 4B) shows that this immunostrip test

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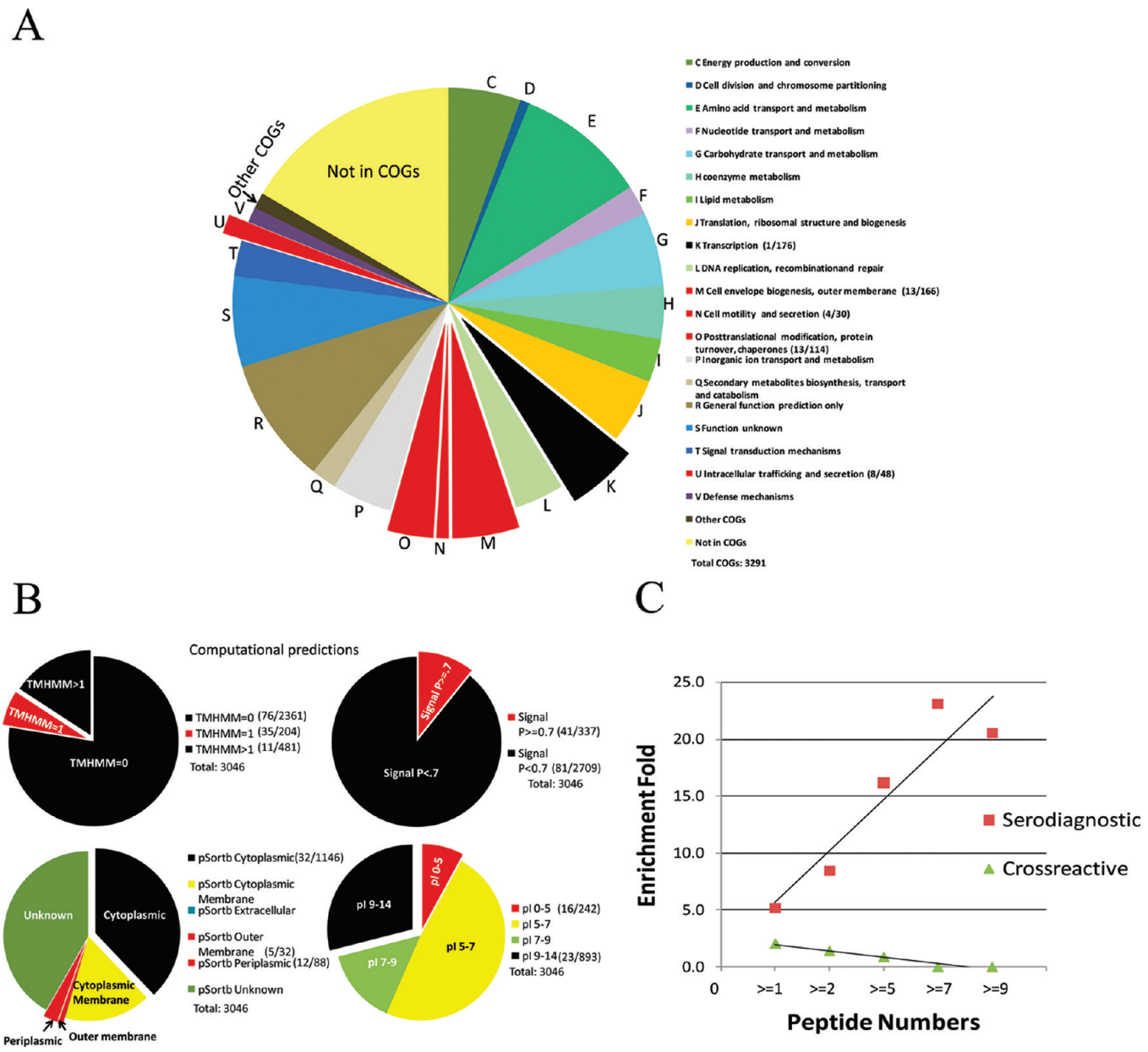

Figure 5. Overview of an enrichment analysis of *Bm* proteome classified with COGs and computational predictions categories. (A) Overview of *Bm* proteome classified with a total of 3291 COGs. The size of each slice represents the number of proteins assigned to each COG category. Proteins classified with COGs-U, M, N, O categories are significantly overrepresented in serodominant antigens and shown in extracted red. COG-K proteins are significantly underrepresented in serodominant antigens are shown in extracted black. COG categories with <5 proteins were not included in the figure (COGs-B, W). (B) Computational predictions for the *Bm* proteome are shown. Significant over- and under-representations are shown in extracted red or black, respectively. Numbers of serodominant antigens within these categories/number of total proteins within these categories are in parentheses in the legend. Results of the complete COG analysis and computational predictions are in Table S3 (Supporting Information). (C) Correlation between enrichment fold and peptide numbers detected by Mass Spectrometry. As peptide numbers for serdiagnostic antigens increase, enrichment fold for serodiagnostic antigens also increases. In contrast, enrichment fold for cross-reactive antigens decreases as peptide numbers for cross-reactive antigens increase.

yielded a high AUC of 0.97, with sensitivity rate of 96.7% and specificity rate of 96.7%.

Comparing Blood Culture and Serological Testing by Agglutination

Serological tests are routinely used to diagnose brucellosis. While the qualitative agglutination test (Rose Bengal) is ~90% sensitive for screening for acute/subacute brucellosis,[50,51] quantitative agglutination testing (e.g., tube agglutination test (TAT)) with titer typically greater than 1/160 used as a more precise diagnostic criterion, can also be used to diagnose relapse. Often blood cultures are negative at the time of initial presentation or relapse, possibly because of low levels of bacteremia or antecedent use of antibiotics. Without being able to ascertain whether the sera used in this study were obtained from patients who might have taken antibiotics prior to blood cultures, we queried our data set to

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Table 1. Enrichment Analysis for *B. abortus* Homolog Proteins Expressed during *in vitro* Growth As Detected by Mass Spectrometry[a]

	proteins		serodominant			serodiagnostic			cross-reactive	
B. abortus homologs expression	on chip	hits	FoldEnrich	*p*-value	hits	FoldEnrich	*p*-value	hits	FoldEnrich	*p*-value
Expressed and detected with at least 1 peptide	356	41	**2.9**	5.28E-11	20	**5.2**	1.87E-11	21	**2.0**	0.001
Expressed and detected with at least 2 peptides	175	23	**3.3**	2.04E-07	16	**8.4**	3.56E-12	7	1.4	0.353
Expressed and detected with at least 5 peptides	40	8	**5.0**	1.38E-04	7	**16.2**	1.30E-07	1	0.9	1.000
Expressed and detected with at least 7 peptides	16	4	**6.2**	3.07E-03	4	**23.1**	1.90E-05	0	0.0	1.000
Expressed and detected with at least 9 peptides	9	2	**5.5**	4.76E-02	2	**20.5**	3.91E-03	0	0.0	1.000
Not expressed	2690	81	**0.8**	5.28E-11	13	**0.4**	1.87E-11	68	**0.9**	0.001
Total ORFs	**3046**	**122**			**33**			**89**		

[a] Significant enrichment or underrepresentation for certain features are underlined and bold.

determine whether sera from blood culture-positive, Rose Bengal positive brucellosis patients (gold standard diagnosis) vs blood culture-negative, Rose Bengal positive (TAT not done) subjects (presumptive positive, unconfirmed) recognized different proteins on the protein microarrays. Sera from blood culture-negative, Rose Bengal positive subjects reacted with 10 *B. melitensis* antigens much more strongly than blood culture-positive, Rose Bengal positive individuals (Figure S3, Supporting Information). This unique set of 10 antigens was distinct from the 33 antigens that distinguish culture positive cases from Peruvian naives. (Table S2, Supporting Information). These results indicate that protein microarray analysis was able to delineate distinct antibody profiles in blood culture positive and negative patients, which has the potential to identify antigens capable of differentiating active acute/subacute brucellosis from previously exposed patients. These findings may suggest novel biological interactions between bacteria and host that need to be further explored because culture negativity/Rose Bengal seropositivity might be associated with some biological difference between human hosts, perhaps an immune response that might lower bacterial loads to levels difficult to culture from blood. Alternatively, it is possible that antibiotic use might lead to an altered host immune response to killed bacteria that no longer would subvert the host immune responses (typical of intracellular pathogens such as *Brucella*).[52−54]

Enrichment Analysis

To better understand the determinants of antigenicity in bacteria, we performed a functional enrichment analysis of the serodominant and serodiagnostic antigens identified in this study. *Bm* proteins are annotated with NCBI Clustered Orthologous Group (COG) functional category. A total of 543 proteins are unassociated with COGs, and 39 proteins are associated with COGs but have not been assigned to a category, shown as "Other COGs". Some proteins have multiple COG assignments so there are 3,291 COGs in total for 3046 proteins on chip.

Proteins with predicted COG- U function, involved in intracellular trafficking and secretion, were 6.2- fold enriched in serodiagnostic antigens and 4.3- fold enriched in serodominant antigens. The enrichment was significant with p value <0.05. Proteins in the COG-M category, cell envelope biogenesis and outer membrane, were 2-fold enriched in serodominant antigens (*p*-value 0.01). Proteins in COG-N category, cell motility and secretion, were 3.5-fold enriched in serodominant antigens (p = 0.027). Proteins with predicted COG-O function, posttranslational modification, protein turnover, chaperones, were also significantly enriched at 3.0-fold in serodominant antigens. There was only 1 serodominant antigen out of 176 COG-K category proteins, predicted to be involved in transcription and identified as serodominant, which was 0.1- fold enrichment and significantly underrepresented with p value of 0.014 (Figure 5A, Table S3A, Supporting Information).

We also looked at enrichment of antigens using computationally predicted features. As shown in Figure 5B and Table S3B (Supporting Information), proteins with 1 transmembrane domain were significantly enriched in serodominant, serodiagnostic and cross-reactive antigens groups, with enrichment fold at 4.3, 5.0, and 4.0, respectively. Proteins lacking transmembrane domains were significantly underrepresented. Interestingly, proteins with more than 1 transmembrane domain were also significantly underrepresented among the serodominant and cross-reactive antigens. Proteins with predicted signal peptides (SignalP score > 0.7) were significantly enriched in all three groups at a range of 2.8- to 3.6-fold. Conversely, proteins without signal peptides (SignalP score < 0.7) were significantly underrepresented. pSortb predicted 4 serodiagnostic and 5 serodominant outermembrane antigens, resulting in 11.5- and 3.9-fold enrichment, respectively. pSortb periplasmic proteins were also significantly enriched, from 3.1- to 4.2-fold. However, pSortb cytoplasmic proteins were 0.5- to 0.7-fold underrepresented in serodominant and serodiagnostic antigens groups. We also found that proteins with isoelectric points lower than 5 were 1.7- to 2.0-fold enriched in serodominant and cross-reactive antigen groups, whereas, proteins with pI = 9 to 14 were 0.6- and 0.5-fold underrepresented in these two groups.

Another potential feature that could account for protein antigenicity is the level of *in vivo* expression; proteins expressed at a high level *in vivo* would be expected more likely to be seen by the immune system than proteins expressed at a low level. To test this hypothesis, we compared the group of antigenic *B. melitensis* proteins with an existing data set of expressed protein peptide data from a very closely related species, *B. abortus*.[23,35,36] In this study, cytosolic proteins and cell envelope proteins from *B. abortus* in the log phase of *in vitro* growth, were extracted and subjected to LC−Mass spectrometry (MS) analysis as described in Materials and Methods. The number of different peptides identified for each protein was listed in Table S4 (Supporting Information). Two-hundred forty proteins were exclusively expressed in the cytosol of *B. abortus* with 1 to12 detected peptides, and 53 proteins exclusively expressed in the cell envelope of *B. abortus* with 1 to 15 detected peptides, and 80 overlapping proteins expressed in both conditions. Of these, 356 proteins

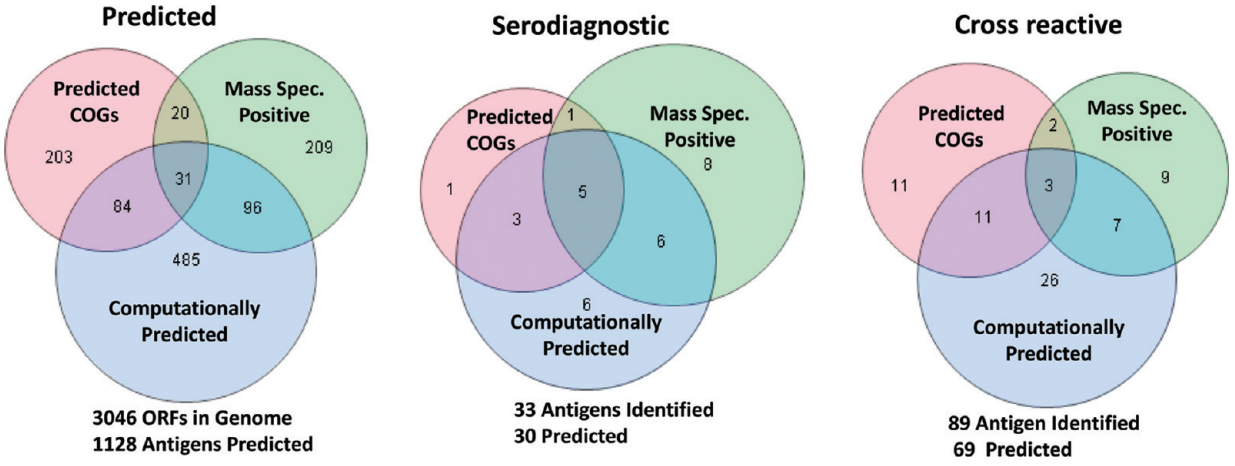

Figure 6. Venn diagram of numbers of antigens with categorized enriching features. Enriching features are classified into three categories: (1) COGs-UMNO, for proteins assigned to one or more of these four COGs; (2) Computational predictions with enriching features, for proteins predicted to have one or more of the following features, that is, TMHMM = 1, SignalP > 0.7, pSort Outermembrane, pSort Periplasmic, or p*I* < 5, and (3) Protein expression as detected by peptide presence in Mass Spectrometry. Separately shown are numbers of antigens with enriching features, including all such 1128 antigens on chip, 99 serodominant, and 30 serodiagnostic antigens. Numbers of antigens having more than one category of enriching features are also presented in the overlapping region of such categories.

Table 2. Enrichment Summary for Antigens with Categorized Enriching Features[a]

	whole genome (3046 on chip)		cross-reactive (89 total)		serodiagnostic (33 total)	
enriching features	predicted	% of total	predicted	% of total	predicted	% of total
COGs-U, M, N, O	338	11%	27	30%	10	30%
Computationally Predicted	696	23%	47	53%	20	61%
Mass Spectrometry Positive (MS+)	356	12%	21	24%	20	61%
COGs + Predicted	919	30%	60	67%	22	67%
COGs + Predicted + MS+	1128	37%	69	78%	30	91%
Predicted + MS+	925	30%	58	65%	29	88%

[a] Enriching features are classified into three categories: (1) COGs-UMNO, for proteins assigned to one or more of these four COGs; (2) Computational predictions with enriching features, for proteins predicted to have one or more of the following features, i.e., TMHMM = 1, SignalP > 0.7, pSort Outermembrane, pSort Periplasmic, or p*I* < 5; and (3) Protein expression as detected by mass spectrometry. Numbers of overlapping antigens for two or three categories are shown in Figure 6.

were printed on our proteome chip. Not surprisingly, expression was a significant enriching feature with 5.2-fold enrichment among serodiagnostic antigens (p-value 1.87×10^{-11}), and 2.9-fold in serodominant antigens (p-value 5.28×10^{-11}) (Table 1). Interestingly, the fold enrichment was directly proportional to the number of peptides detected (Figure 5C). There was 8.4- fold enrichment of serodiagnostic antigens in proteins detected with more than 1 peptide (p-value 3.56×10^{-12}), 16.2- fold in proteins detected with 5 or more peptides (p-value 1.30×10^{-7}), 23.1- fold enrichment in proteins detected with 7 or more peptides (p-value 1.90×10^{-5}), and 20.5-fold enrichment in proteins detected with 9 or more peptides (p-value 3.91×10^{-3}). Conversely, proteins that were not identified by MS were significantly underrepresented at 0.4-fold among the serodiagnostic and 0.8-fold among serodominant antigens. The data suggests that protein expression level is an important factor contributing to antigenicity of protein antigens. This was also supported by data in other experimental systems from different research groups.[55]

There are a total of 10 enriching features that fall into 3 categories: (i) functionally annotated COGs U, M, N and O, (ii) computationally predicted features (TMHMM = 1, SignalP > 0.7, pSort Outermembrane, pSort Periplasmic, and p*I* < 5), and (iii) MS evidence of expression. The Venn diagrams summarize the number of enriched proteins found in each of these 3 categories, showing overlap among the proteins found in each category (Figure 6). There are 338 proteins in the enriched COG categories, 696 computationally predicted proteins, and 356 proteins that were positive by MS (Table 2). Accounting for the overlap between categories, 1128 proteins in all enriched categories represented 37% of the proteome. The microarray results empirically identified 33 serodiagnostic antigens and 89 cross-reactive antigens. COGs accounted for 30% of the serodiagnostic hits, the computationally predicted features accounted for 61% of the hits, and MS positive proteins contained 61% of the hits. All together the three categories account for 37% of the proteome and include 91% of the serodiagnostic antigens. But there is only 1 unique serodiagnostic COG antigen that is not represented in either the computationally predicted or MS+ categories (BMEI1060, Table S1, Supporting Information). So by combining the pool of computationally predicted and MS+ proteins representing 30% of the proteome, 88% of the serodiagnostic antigens can be predicted.

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DISCUSSION

Today with increasing efficiency, accuracy and speed we access completed genome sequences from thousands of infectious microorganisms. However, systems- level studies to understand the complete network of antibody responses have not been widely performed. This work was motivated by the disturbing lack of a detailed scientific understanding of the antibody responses to infectious diseases. We have been taking a systems biology approach to account for all of the antibodies that develop after exposure to infectious microorganisms and to identify specific antibody signatures associated with each disease, in order to understand the molecular basis for antigen selection by the immune system and to predict serodiagnostic and vaccine antigens targets.

This study is the first full proteome-wide serological analysis of *B. melitensis*. Our previous study identified a set of serodiagnostic antigens from a randomly selected half of the *B. melitensis* proteome. However, an important difference between the present study and this previous report is that analysis of the complete proteome not only has allowed us to delineate additional serodiagnostic antigens but, more fundamentally, also provided the basis for a rigorous, comprehensive and quantitative determination of basic biological characteristics of the entire set of the serodominant antigens on a genomic level.

The current standard serological screening assay, an agglutination test that uses tinted, killed bacteria as antigen (Rose Bengal) is based primarily on identification of antibodies to LPS in patient serum. In the present microarray study, we confirmed LPS reactivity (with purified LPS spotted onto the microarray), and identified novel protein antigens. The signature antigens were validated using traditional Western blots. Although antibody responses against all 33 protein antigens used together can predict disease with 92% accuracy, using the serological responses against the top 3 individual antigens together improves accuracy to >98% for diagnosis of acute human brucellosis. One limitation is that the humans studied here only had acute or subacute brucellosis-fever but not focal or chronic disease. Future studies are needed to assess suspected cases that are agglutination test negative, for example chronic neurobrucellosis or focal disease such as orchitis or vertebral osteomyelitis.

Eleven of the 33 serodiagnostic antigens identified in this study were also identified on the pilot array[5] (Table S1, Supporting Information). Our findings are in good agreement with published studies that identified well characterized antigens from other *Brucella spp*, including Bp26 (BMEI0536),[20,25,31,56] HtrA/DegP (BMEI1330),[28] Omp16 (BMEI0340),[29] the chaperonin GroEL protein (BMEII1048) and Omp 10 (BMEII0017).[21,57] In addition to the well characterized antigens, we also identified 21 novel serodiagnostic antigens on the current array, including top antigen hypothetical protein BMEI0805. Two pyruvate dehydrogenase complex molecules and the associated acetyl CoA hydrolase, which together form a large multimeric structure consisting of 60 subunits, are also differentially recognized; this large enzyme complex is found in most bacteria and is frequently a target of immune recognition for other infections.[6,58]

Protein microarrays enable enrichment analyses to identify proteomic features that are enriched in the immunodominant antigen set, and development of protein antigenicity prediction tools based on enriched features. The prediction tools then can be applied on a high-throughput scale to existing or new proteomes to identify key antigenic proteins that may have serodiagnostic or protective characteristics. Enrichment analysis identified 10 proteomic features that are enriched in the serodiagnostic signature antigens. The proteomic features fall into 3 categories: (i) COG annotations, (ii) computationally predicted proteomic features, and (iii) proteins with MS evidence of expression in viable organisms. No single proteomic feature or category of features is sufficient to identify all these signature antigens. Our data suggests that cloning of 37% of genome with these enriching features would reveal more than 90% of serodiagnostic antigens. We have classified the reactive immunodominant antigens for numerous agents and have consistently found these features predict antigenicity.[6,7,10,59]

These results describe the relationship between antigenicity and *in vivo* expression of individual proteins. In our study, we utilized the expression of proteins quantified by MS of a closely related strain, *B. abortus* during *in vitro* growth. We found that mass spec positive antigens were significantly enriched among serodiagnostic antigens but not among the cross- reactive antigens. This validates the classification of the serodiagnostic antigens being derived from actively replicating organisms, and validates the classification of cross-reactive antigens being derived from previous exposure to nonbrucellosis infections. Although the number of peptides observed per protein has been applied to estimating protein abundance,[60−62] we are aware that mass spectra of cultured organisms may not reflect the expression during infection *in vivo*. First, nutrients are not limiting in log phase growth *in vitro*, whereas *in vivo*, *Brucella* is likely to be within a nutrient-limited intracellular environment.[63] Second, the MS method may not be particularly quantitative, especially for membrane proteins. Our protein array technology is also able to provide strong evidence of the comprehensive set of proteins expressed *in vivo* within a mammalian host by *B. melitensis,* by virtue of their exposure to the host immune system. The expression and abundance of proteins during *in vivo* growth merits further examination by other novel technologies, such as measurement of transcript abundance from RNA-seq ("deep sequencing") technique.[64]

The *B. melitensis* immunoproteome comprises 1464 antigens that are significantly reactive in at least one of the individuals in this study. Only 122 serodominant antigens are significantly recognized by most of the individuals. Answers to questions of why and how the immune system focuses on 4% of the potential target antigens are not yet apparent from this work. One might expect that an immune response against a larger collection of antigens could result in a more effective immune response attack against the infectious agent. But antibody responses against thousands of antigens from hundreds of clinical infections could accumulate during a lifetime, leading to cross reactivity against autologous antigens and autoimmune chaos. This could provide evolutionary selection pressure favoring a more focused response to infection. The observation that dozens of organism- specific antibody responses develop after *B. melitensis* infection is consistent with similar observations from other infectious agents, and has implications for subunit vaccine discovery and development. Mimicking the natural response to infection could be considered a viable strategy for vaccine development but most subunit vaccines aim to derive protection from immunization with only a single antigen. Attenuated or killed whole organism vaccines produce an antibody reactivity profile against dozens of antigens more similar to natural infection.[6]

This systematic genome scale analysis of human antibody responses against *B. melitensis* proteins provides a top hit list of antigens worthy of assessing for improved diagnostics, and

furthermore, enables development of a predictive model of proteomic features that determine whether a protein is antigenic and produces antibodies that confer protection. This systems biology approach provides an empirical basis for understanding the breadth and specificity of the immune response to *B. melitensis* and a new framework for comparing the humoral responses against other organisms.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Conflict of InterestP.L.F. and D.H.D. have patent applications pertaining to this work and own stock in Anitigen Discovery Inc. that has licensed the technology. X.L. is an inventor on these patent applications and is employed at Anitigen Discovery Inc. W.J.W.M. is employed at Anitigen Discovery Inc.

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